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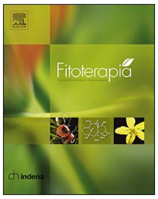

Extraction of carboxylic acid-containing diterpenoids from *Dodonaea viscosa* via pressurised hot water extraction

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ABSTRACT

Pressurised hot water extraction (PHWE) was employed to effect the extraction of two carboxylic acid-containing *ent*-labdane diterpenoids from *Dodonaea viscosa*. The different extraction profile provided by PHWE in this case suggests that this recently developed method also has applications as a complementary tool for natural products extraction.

1. Introduction

Dodonaea viscosa (L.) Jacq. (Sapindaceae) represents a ubiquitous species of shrub found throughout Australia and five other continents [1]. Genetic evidence has shown that *Dodonaea viscosa* evolved in Australia in the late Pliocene or early Pleistocene [1]. *D. viscosa* has been utilised in traditional medicine for the treatment of conditions such as skin infections, rheumatism, dermatitis and haemorrhoids [2,3]. Numerous phytochemical studies of *D. viscosa* have been undertaken leading to the isolation of a range of clerodane diterpenoids, *ent*-labdane diterpenoids, flavonoids and triterpenoids [4]. *D. viscosa* ssp. *spatulata* is endemic to Australia and is found throughout the southern regions of the continent, including Tasmania [1]. Recently, we reported the isolation of nine different *ent*-labdane diterpenoids (**1**–**9**) from a specimen of *D. viscosa* ssp. *spatulata*, seven of which were unreported (**1**,**2**,**4**,**5**,**7**–**9**) (Fig. 1) [4]. In addition to Sapinadaceae, *ent*-labdane diterpenoids have been isolated from numerous plant families including, Acanthaceae [6,7], Asteraceae [8], Euphorbiaceae [9] and Meliaceae [10]. A diverse array of biological activity has been reported for *ent*-labdane diterpenoids including anti-cancer [5], anti-inflammatory [5,6], hepato-protective [7], anti-fungal [8] and anti-bacterial [11] properties.

In our previous study on *Dodonaea viscosa* ssp. *spatulata*, the resinous leaf material was soaked in diethyl ether for ≤ 1.0 h to provide an extract from which the above-mentioned *ent*-labdanes **1**–**9** were isolated after flash column chromatography (carboxylic acids **8** and **9** were isolated as their respective methyl esters after derivatization) [4]. Despite the large number of diterpenoids that were isolated employing this approach, we speculated that the extraction with diethyl ether for short periods of time (1 × 0.5 h) might not be exhaustive. Although, we observed that soaking the leaf material multiple times (2 × 0.5 h) did not change the extraction profile.

Interestingly, in an initial experiment, we observed that the extraction of *Dodonaea viscosa* ssp. *spatulata* employing pressurised hot water extraction (PHWE) appeared to provide a different extraction profile [4]. We have previously employed our novel, rapid PHWE method for the efficient extraction and isolation of shikimic acid (**10**) from star anise [12], polygodial (**11**) from *Tasmannia lanceolata* [13], eugenol (**12**) from cloves [14], asperuloside (**13**) from *Coprosma quadrifida* [15], and five coumarin natural products including seselin (**14**), epoxysuberosin (**15**), and heraclenin (**16**) from various *Correa* (Rutaceae) species (Fig. 2) [16]. These studies demonstrated that PHWE could be utilised to effectively extract natural products that featured a relatively broad polarity range. Moreover, compounds containing sensitive functional groups, including aldehydes, epoxides, glycosides, and potentially epimerisable stereogenic centres were tolerated by the extraction process. With this in mind, we sought to extensively evaluate the potential of PHWE to serve as a complementary extraction tool for the isolation of carboxylic acid-containing *ent*-labdane diterpenoids from *Dodonaea viscosa* ssp. *spatulata*.

2. Experimental

2.1. General

NMR experiments were performed on a Bruker Avance III NMR spectrometer operating at 600 MHz (^{1}H) or 150 MHz (^{13}C). The deuterated solvent used was $CDCl_3$ and chemical shifts were recorded in ppm. Spectra were calibrated by reference to the residual solvent peak at δ_H 7.26 and δ_C 77.16 ppm. Coupling constants (J) were recorded in

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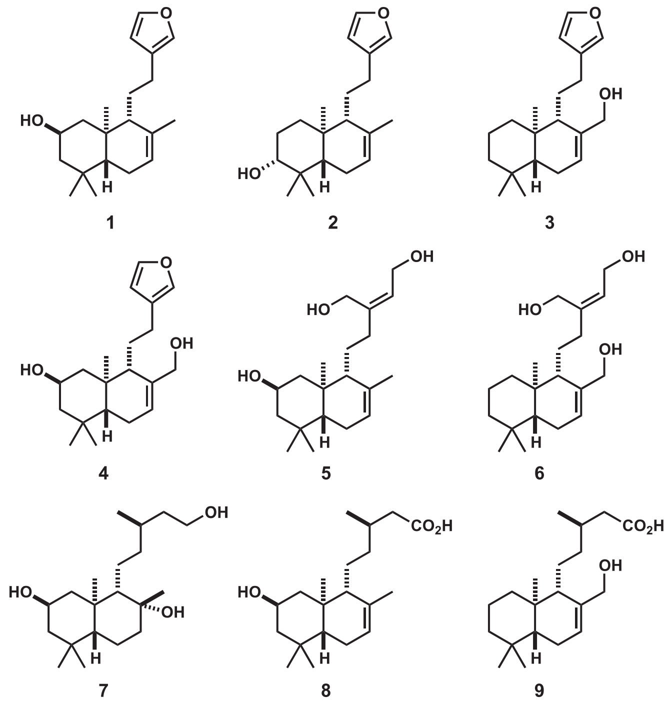

Fig. 1. *ent*-Labdane diterpenoids **1**–**9** isolated from *D. viscosa* ssp. *spatulata*. Carboxylic acids **8** and **9** were isolated as their respective methyl esters after derivatisation.

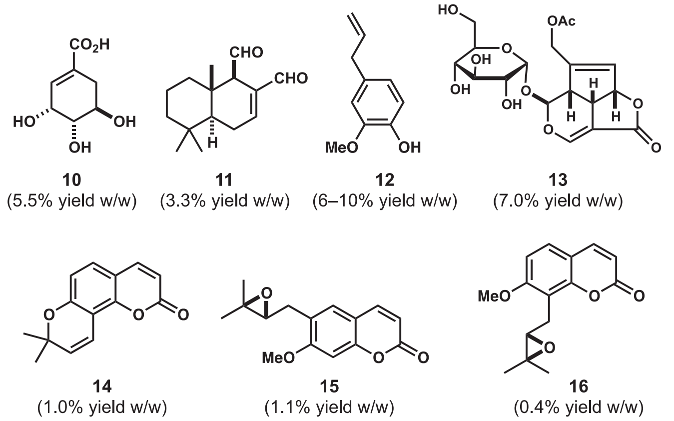

Fig. 2. Natural products previously isolated via PHWE: shikimic acid (**10**), polygodial (**11**), eugenol (**12**), asperuloside (**13**), seselin (**14**), epoxysuberosin (**15**) and meranzin (**16**).

Hz. HRESIMS analyses were conducted on a Thermo-Scientific LTQ-Orbitrap using a syringe pump operated at 3 µL/min, and full scan data acquired in positive ionisation mode using an electrospray voltage of 5 kV. MS samples were prepared in $CHCl_3$. Polarimetry was performed with a Rudolph Research Analytical Autopol III automatic polarimeter operating at 589 nm using a 0.5 dm cell. Infrared spectrometry was performed on a Shimadzu FTIR 8400 s spectrometer. A thin film of material was deposited on NaCl plates following evaporation of CH_2Cl_2, $CHCl_3$ or $CDCl_3$.

Analytical grade solvents were used and purified by standard laboratory procedures. Thin-layer chromatography (TLC) was performed using Merck silica gel 60-F_{254} plates. Developed TLC plates were visualised by UV absorbance (254 nm) or through application of heat to a plate stained with cerium molybdate $\{Ce(NH_4)_2(NO_3)_6, (NH_4)_6Mo_7O_{24}{\cdot}4H_2O, H_2SO_4, H_2O\}$. Manual flash column chromatography was performed with flash grade silica gel (60 µm) and the indicated eluent in accordance with standard techniques [17]. Automated flash column chromatography was performed with a Grace Reveleris X2 flash column chromatography system with 40 µm silica gel cartridges and the indicated eluent gradient. Automated reversed-phase flash column chromatography was performed with a Grace Reveleris X2 flash column chromatography system with C-18 capped silica gel cartridges. Plant material was ground using a Sunbeam spice/coffee bean grinder. PHWE was undertaken employing a Breville Espresso Machine Model 800ES.

2.2. Plant material

Leaves and stems were obtained from a single specimen of *Dodonaea viscosa* ssp. *spatulata* at the University of Tasmania, Sandy Bay campus (location: 42.904134S, 147.323871E). A voucher specimen (no. HO588356) has been lodged with the Tasmanian Herbarium and identified by Dr. Miguel de Salas. The plant material was dried in an oven maintained at 45 °C for 24 h. The leaves were finely ground in a spice grinder prior to extraction.

2.3. Extraction and isolation

Dried and finely ground *D. viscosa* ssp. *spatulata* leaves (10 g) were mixed with sand (4 g), placed into the portafilter (sample compartment) of an unmodified espresso machine and extracted using 35% v/v $EtOH/H_2O$ (200 mL of a hot solution; ~2 min). This was repeated a further six times with equal portions of ground leaf material (70 g in total). The ensuing extracts were then combined and concentrated to dryness under reduced pressure on a rotary evaporator to remove both EtOH and H_2O (45 °C bath temperature) to provide a crude residue (31.2 g, ~45% w/w). Acetone (60 mL) was added to the ensuing residue and, after 30 min, the mixture was filtered to remove insoluble material and the filtrate collected. The ensuing insoluble material was extracted in this fashion a further three times until the sample no longer contained *ent*-labdane diterpenoids (as judged by TLC and ^1H NMR analysis). The combined organic extracts were concentrated under reduced pressure to provide a crude residue (9.47 g, ~14% w/w). The ensuing residue was adsorbed on silica/Celite® (~1:1 ratio) then subjected to automated flash column chromatography (0 → 100% EtOAc/hexanes, 10 min; 0 → 10% $MeOH/CH_2Cl_2$, 8 min; 10 → 20% $MeOH/CH_2Cl_2$, 4 min) to provide two main fractions (as judged by TLC analysis): fraction A (222 mg) and fraction B (2.17 g). Fraction A was subjected to flash chromatography to provide a mixture of the previously isolated furano-diterpenoids **1** and **2** (29 mg in total, $<$ 0.04% w/w) [4]. Fraction B was subjected to automated flash column chromatography (0 → 30% EtOAc/hexanes, 11 min; 30–100% EtOAc/hexanes, 4 min; 0 → 3.3% $MeOH/CH_2Cl_2$, 3.5 min; 3.3 → 10% $MeOH/CH_2Cl_2$, 1.5 min) to provide a residue (1.64 g). A portion of this sample (378 mg) was then adsorbed on C-18 capped silica and subjected to automated reversed-phase flash column chromatography (25 → 100% $MeCN/H_2O$, 13 min) to provide carboxylic acid **8** (135 mg, 0.84% w/w) and **9** (5 mg, 0.03% w/w). Compound **8**: $[\alpha]_D^{20}$ + 6.4 (*c* 0.97, $CHCl_3$); IR (NaCl) 3379, 2959, 2928, 2853, 1709, 1464, 1379, 1279, 1159, 1024, 912, 733 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 5.39 (m, 1H), 3.87 (tt, *J* = 11.5, 3.9 Hz, 1H), 2.38 (dd, *J* = 14.9, 6.2 Hz, 1H), 2.18–2.14 (m, 2H), 2.00–1.96 (m, 2H), 1.83 (m, 1H), 1.76 (m, 1H), 1.67 (s, 3H), 1.64 (brs, 1H), 1.59–1.48 (m, 2H), 1.26–1.12 (m, 4H), 1.00 (d, *J* = 6.7 Hz, 3H), 0.91 (s, 6H), 0.80 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 178.0, 135.2, 122.3, 65.4, 55.4, 51.3, 49.6, 48.5, 41.1, 39.2, 38.9, 34.9, 33.3, 31.1, 24.4, 23.8, 22.9, 22.1, 20.1, 14.6; HRESIMS *m/z* $[M + Na]^+$ calcd for $C_{20}H_{34}O_3Na$ 345.2400; found 345.2395. Compound **9**: $[\alpha]_D^{20}$ + 11.2 (*c* 0.25, $CHCl_3$); IR (NaCl) 2957, 2924, 1707, 1463, 1382, 1364, 1209; ^1H NMR (600 MHz, $CDCl_3$) δ 5.75 (m, 1H), 4.13 (d, *J* = 12.2 Hz, 1H), 3.97 (d, *J* = 12.2 Hz, 1H), 2.36 (dd, *J* = 15.1, 6.8 Hz, 1H), 2.20 (dd, *J* = 15.1, 7.5 Hz), 2.05 (m, 1H), 1.97–1.91 (m, 2H), 1.85 (m, 1H), 1.78 (brs, 1H), 1.70–1.63 (m, 2H), 1.57–1.51 (m, 2H), 1.47–1.40 (m, 2H), 1.22–1.13 (m, 4H), 1.00 (d, *J* = 6.7 Hz, 3H),